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Resolution of the hemes of hydroxylamine oxidoreductase by redox potentiometry and optical spectroscopy

Roger C. Prince, Christian Larroque+ and Alan B. Hooper*

Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA 19104, USA, +INSERM U128, Montpellier 34033, France and **Department of Genetics and Cell Biology, University of Minnesota, St. Paul, MN 55108, USA*

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Optical spectroscopy combined with redox potentiometry has resolved the hemes of hydroxylamine oxidoreductase into 6 thermodynamically distinct classes. There are apparently 4 classes of heme c_{553}, with E_{m7}-values of 295 mV, 10 mV, −190 mV and −390 mV, present in a stoichiometry of 1:1:2:1; two equivalents of heme c_{559}, E_{m7} O mV, and one of heme P-460, an unusual chromophore, with E_{m7} −260 mV.

Hydroxylamine oxidoreductase *Redox potentiometry* Nitrosomonas *Cytochrome*

1. INTRODUCTION

The oxidation of NH_4^+ to NO_2^-, a key part of the nitrogen cycle of the biosphere, is mainly catalyzed by soil autotrophs of the genus *Nitrosomonas*. These organisms contain the remarkable enzyme hydroxylamine oxidoreductase (HAO), which catalyzes the 4 electron dehydrogenation of NH_2OH to form HNO_2 (unpublished). In addition to a dehydrogenase site, HAO has at least one oxygen-activating site [1], and in line with this complicated chemistry, has a complicated complement of redox centers. Optical, EPR and Mossbauer spectroscopy reveal that the enzyme is an $\alpha_3\beta_3$ aggregate ($M_r \sim 200\,000$) of a large protein containing 6 *c*-type hemes and one heme P-460, and a small monoheme cytochrome *c* [2,3]. The $\alpha_3\beta_3$ aggregate thus contains 24 hemes! P-460 is found only in the ammonia oxidizing autotrophic bacteria, has an absorption maximum, in the reduced form, at 463 nm [4], and has been shown by Mossbauer spectroscopy to be a heme derivative [5]. It is involved in both the dehydrogenase [6] and oxygen reactivity [1] of the enzyme. The *c*-type hemes fall into two optically distinct groups, with α-band maxima at 553 and 559 nm. The *c*-hemes are partially reduced by NH_2OH under anaerobic conditions, where P-460 remains oxidized [7]. EPR spectra of oxidized HAO reveal at least 3 types of low-spin hemes together with a high-spin species tentatively identified as P-460 [7,8].

We here report a redox potentiometric titration of the enzyme, assayed by optical spectroscopy. It reveals 4 thermodynamically distinct c_{553} hemes, one class of c_{559} hemes, and one class of P-460 heme.

2. MATERIALS AND METHODS

Hydroxylamine oxidoreductase was prepared from *Nitrosomonas europaea* as in [2]. The redox titrations were carried out as in [9] in an anaerobic cuvette containing approximately 1.2 μM hydroxylamine oxidoreductase, 100 mM KCl, 20 mM *N*-morpholino propane sulfonic acid, 1 mM $MgCl_2$ (pH 7.0) with 50 μM of each of the following redox mediators: 2,3,5,6-tetramethylphenylenediamine (E_{m7} 275 mV); *N*-methylphenazonium methosulfate (E_{m7} 85 mV); *N*-ethylphenazonium ethosulfate (E_{m7} 65 mV); phenazine (E_{m7} −125 mV);

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2-hydroxy-1,4-naphthoquinone (E_{m7} −145 mV); 2-hydroxy-1,4-anthraquinone (E_{m7} −205 mV); benzyl viologen (E_{m7} −350 mV); and methyl viologen (E_{m7} −430 mV) [10].

Spectra were recorded with a Perkin-Elmer 557 double-wavelength double-beam spectrophotometer, using 590 nm as the reference wavelength, in an external cell compartment that allowed stirring the cuvette from the bottom. The EMI photomultiplier (model RFI/S) was shielded so that the stirrer operated throughout the experiment.

3. RESULTS

Fig.1 shows a redox titration of the hemes of HAO. The data represent the sum of 5 different experiments involving two separate preparations of the enzyme, but not all experiments covered the entire range of ambient potential. The low potential region (−450 mV to +100 mV) was usually titrated in an oxidative direction, starting with the fully reduced sample, although some points were also measured in reductive titrations. The points for the high potential region were obtained in both oxidative and reductive titrations, starting at either +100 mV or +500 mV. Each point reflects the measurement of heme α-band peak height, or absorption at 460 nm, measured from a complete difference spectrum from 400 to 600 nm. The absorbance of reduced P-460 was estimated as ΔA 460 nm + 0.43 Δ553 nm in order to compensate for the decreased absorbance at 460 nm due to reduction of the *c* hemes. Fig.2 shows some representative spectra revealing the 3 groups of hemes. At high-E_h (+181 minus +374 mV) the high potential heme c_{553} (E_{m7} +295 mV) is seen. In the intermediate range (−126 minus +181 mV), another heme c_{553} (E_{m7} +10 mV), and heme c_{559} (E_{m7} 0 mV) are revealed, and the low potential region (−430 minus −126 mV) reveals more heme c_{553} (E_{m7} −190 and −390 mV) and heme P-460 (E_{m7} −260 mV). The sloping baselines in this figure reflect absorbance changes of the redox dyes added to mediate electron equilibration between the hemes and the electrode, and illustrate the importance of measuring complete spectra for the redox titrations.

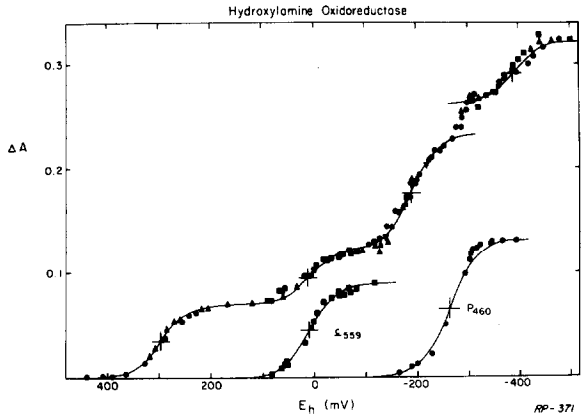

Fig.1. Redox titrations of the *c* hemes and P-460 of hydroxylamine oxidoreductase. The solution contained the components listed in section 2. The different symbols represent different titrations. The major line represents the size of the α-band of hemes c_{553}, the two other lines the titrations of heme c_{559} and P-460 as indicated. The gap in the lines drawn through the heme c_{553} data around −260 mV reflect the possibility that changes in this spectral region accompany the reduction of P-460 (e.g., [13]). The data do not allow a precise estimate of the contribution of this component, and ignoring it would not significantly affect the calculated E_m-values of the heme c_{553}.

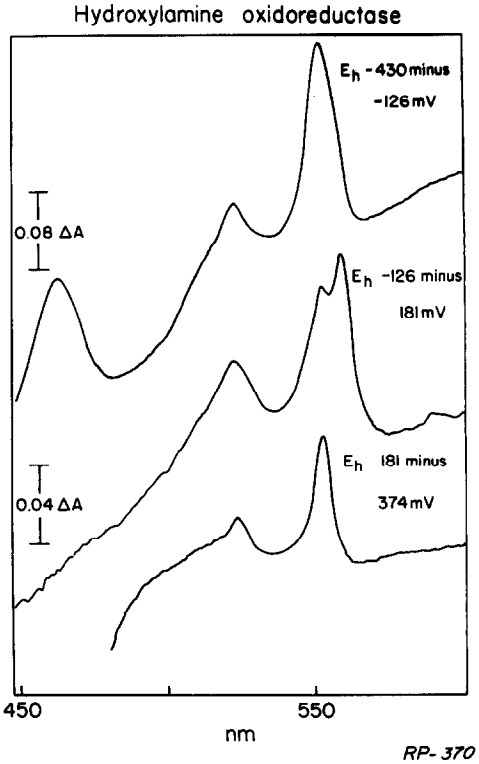

Fig.2. Representative spectra of the titrations of fig.1.

4. DISCUSSION

The redox titrations reported here reveal 4 thermodynamically distinct classes of heme c_{553}, and one class each of heme c_{559} and heme P-460. The *c*-type hemes seem relatively simple to interpret: all appear to titrate as one electron redox couples, and if we assume roughly equal extinction coefficients for the different forms, we may assign the 7 *c*-type hemes [1] as follows: hemes c_{553} with E_{m7} of +295 mV; +10 mV; −190 mV and −390 mV in a ratio of 1:1:2:1, together with two equivalents of heme c_{559} with E_{m7} 0 mV. Other interpretations are, of course, possible, for example, the heme c_{553} E_{m7} +10 mV and the heme c_{559} E_{m7} 0 mV might in fact be a single class of cytochrome (at a relative stoichiometry of 3?) with a split α-band, but EPR experiments suggest that this is unlikely ([7,8], unpublished). Some of the hemes c_{553} have unusually low midpoint potentials, but this is not unprecedented. For example, cytochromes c_3 from *Desulfovibrio* species have 4 *c*-type hemes with E_m-values between −250 and −350 mV [12,13].

The heme responsible for the absorption at 463 nm, known as P-460, seems to be unique to hydroxylamine oxidoreductase. Under the conditions used here its E_{m7} is −260 mV; the poor fit to a one-electron titration in fig.1 may be due to the difficulty of resolving this heme, at least at low levels of reduction, for the Soret bands of the *c*-type hemes. On the other hand, P-460 may not be titrating as a simple one-electron component, for the EPR data ([2], unpublished) indicate that the high-spin ferric signal of P-460 is seen only in a narrow range of E_h near the E_m of this heme. The P-460 heme may perhaps be related to the P-450 hemes of mixed function oxidases, and the E_{m7} of −260 mV for P-460 is not very different from the E_{m7} of both bacterial and hepatic microsomal P-450 when the latter are free of substrates (E_{m7} −300 mV, see [14]). It is noteworthy that the E_{m7} of P-450 shows a shift of more than 100 mV upon the binding of substrates, and the value for P-460 reported here might reflect the E_m of the heme modified by the binding of redox dyes.

The midpoint potentials of the *c*-type hemes of hydroxylamine oxidoreductase span a wide range of redox potentials, almost 700 mV. Presumably, this reflects the complicated nature of the redox chemistry involved in the oxidation of hydroxylamine. These reactions are still poorly understood, and the sequence of electron flow among the hemes remains to be elucidated.

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